Interaction of matrix metalloprotease-9 and Zpx in Cronobacter turicensis LMG 23827T mediated infections in the zebrafish model

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Abstract: Bacteria belonging to the genus Cronobacter have been recognized as causative agents of life-threatening systemic infections primarily in premature and low-birthweight neonates. Validation of putative bacterial virulence components as well as host factors potentially involved in the response to infection has been hampered in the past by the availability of suitable neonatal animal models. In the current study, the zebrafish embryo model was employed to study the interaction of the zinc metalloproteinase Zpx present in Cronobacter turicensis LMG 23827T , with the eukaryotic MMP-9, a proteinase that functions to cleave extracellular matrix gelatin and collagen. Cleavage and activation of the human recombinant pro-MMP-9 by zpx-expressing C. turicensis cells were demonstrated in vitro, and the presence and increase of the processed, active form of zebrafish pro-MMP-9 were shown in vivo. We provided evidence that Zpx induces the expression of the mmp-9 but also increases the levels of processed MMP-9 during infection. The involvement of the MMP-9 in induction of the expression of the bacterial Zpx was shown in zebrafish mmp-9 morphant experiments. This study identified MMP-9 as a substrate of Zpx and demonstrated yet-undescribed mutual cross-talk between these two proteases in infections mediated by C. turicensis LMG 23827T .

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The role of host matrix metalloprotease-9 and Zpx in *Cronobacter* spp. mediated sepsis in a morphant zebrafish model

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ABSTRACT

Bacteria belonging to the genus *Cronobacter* (*C.)* have been recognized as causative agents of life-threatening systemic infections primarily in premature, low-birth weight and immune-compromised neonates. Elucidation and validation of putative bacterial virulence components as well as host factors possibly involved in the response to infection has been hampered in the past by the availability of suitable neonatal animal models. In the current study the recently developed zebrafish embryo model was employed to study the interaction of the zinc metalloprotease Zpx present in *Cronobacter* spp. with MMP-9, a protease cleaving extracellular matrix gelatin and collagen. RT-PCR revealed differential expression of the zebrafish *mmp-9* upon infection with *Cronobacter*, as well as an increase of the processed, active form of the MMP-9 over the course of infection. Employing a *C. turicensis* z*px* knock out mutant revealed that the presence of this bacterial protease induces the expression of the *mmp-9* but also increases the levels of processed MMP-9 during infection. Most striking, experiments using a morpholino knock down of zebrafish *mmp-9* suggest involvement of the MMP-9 in induction of the expression of the bacterial protease. This study identified host MMP-9 as a substrate of Zpx and demonstated yet undescribed mutual cross-talk between these two proteases in *Cronobacter* mediated infections.

INTRODUCTION

Cronobacter represents a genus of opportunistic pathogens that can cause severe infections predominantly in pre-term and/or immune compromised neonates for which high fatality rates ranging between 40 and 80% have been reported[1]. Clinical manifestations of infection include necrotizing enterocolitis, septicemia and neonatal meningitis and pneumonia[1]. Current knowledge of the pathogenesis as well as the host response elicited of these bacteria remains to be elucidates, a feature that particularly applies to those host factors that may be involved during infection.

Matrix metalloproteases (MMPs) such as MMP-9, are involved in the migration of inflammatory cells across the extracellular matrix, as well as contributing to tissue remodeling[2,3]. It has been shown that levels of MMP-9 increase rapidly, in blood circulaton of mice and the serum of healthy human volunteers, following the introduction of *E. coli* LPS[4,5]. In addition, it was demonstrated that MMP-9 deficiency protects against mortality in a murine endotoxic shock model, and selective blocking of MMP-9 was suggested as a potential novel therapeutic approach for treatment of sepsis[5]. However, in other studies a protective role of MMP-9 against infections caused by *E. coli* and *Listeria* has been reported and this discrepancy in results was explained by the existance of a well-regulated balance between inflammatory processes, necessary to combat invading pathogens and overshooting inflammation which can result in tissue damage[6,7].

In addition to induction of MMP-9 secretion by the host, the activation of pro-MMP-9 can be triggered by releasing bacterial proteolytic enzymes. Thus activation

of MMP-9 has been reported from serine proteases associated with LPS preparations[8]. *Streptococcus pneumoniae* the major cause of severe pneumonia, secretes a zinc metalloproteinase ZmpC which cleaves MMP-9 *in vitro*. In a murine pneumonia model ZmpC mutants caused 75% less mortality than wild-type pneumococci, confirming a role of ZmpC in pathogenesis[9].

In *Cronobacter*, a zinc metalloprotease Zpx was identified and characterized by Kothary et al.[10] and its role as a virulence factor during *in vivo* infection was confirmed by Eshwar et al[11]. In the latter study, the recently developed zebrafish embryo infection model was used to study sepsis caused by *Cronobacter* [11,12].

In the current study we wanted explore the involvement of the MMP-9 in *Cronobacter* pathogenesis as well to investigate its interaction between *zpx* and MMP-9 *in vivo* using the zebrafish embryo sepsis model.

MATERIALS AND METHODS

Bacterial strains.

The bacterial strains used in this study are listed in Supplemental Table T1. *C. turicensis* LMG 23827^T , is a clinical isolate responsible for the death of two neonates in Zurich 2006[13] and the GFP expressing variant was constructed by Schmid et al.[14]. Construction of the *C. turicensis* LMG 23827^T Δ*zpx* mutant, the complemented mutant *C. turicensis* LMG 23827^T Δ*zpx*/pQE30::*zpx* as well as the mutant carrying the expression vector plasmid pQE30 only is described in detail by Eshwar et al.[11] *C. muytjensii* LMG 51329^{T}dsRED was constructed by transformation of the strain with plasmid pRZT3::dsRED. *E. coli* XI1 blue was transformed either with the plasmid

pQE30 (the original vector) or plasmid pQE30::*zpx* constructed earlier by Eshwar et al. (2016), using standard molecular techniques. Bacteria were grown at 37 °C in Luria-Bertani (LB) broth with shaking (210 rpm) or on LB agar supplemented with the respective antibiotics: ampicillin (100 mg L^{-1}), tetracycline (30 mg L^{-1}) and nalidixic acid (256 mg L^{-1})(Supplemental Table T1).

For microinjection experiments, the bacteria were grown to stationary phase in LB overnight at 37 °C, harvested by centrifugation at 5,000 x *g* for 10 min and washed once in 10 ml of Dubelcco`s phosphate buffered saline (DPBS, Life Technologies.) After a second centrifugation step, the cells were resuspended in DPBS, and appropriate dilutions were prepared in DPBS.

Zebrafish lines and husbandry

Zebrafish (*Danio rerio*) *wik* lines were used in this study. Husbandry, breeding and bacterial inoculum preparation was performed following the procedure described by Eshwar et al.[11] Virulence was assessed by determination of the survival rate (30 embryos per experiment/strain) over 72 (hpi) hours post infection (= 3 days post infection, dpi). The following controls were included: infection with (apathogenic) *E. coli* Xl1 blue, injections with Dulbecco's Phosphate Buffered Saline (DPBS), and non-injected embryos. Microinjections of bacteria were performed using borosilicate glass microcapillary injection needles (Science Products, 1210332, 1 mm O.D. x 0.78 mm I.D.) and a PV830 Pneumatic PicoPump (World Precision Instruments).

The maximum age reached by the embryos during experimentation was 120 hpf (hours post fertilization = 72 hpi) for which no license is required from the

cantonal veterinary office since embryos had not yet reached free feeding stage.

Infection studies

Two-dpf (days post fertilization) embryos were manually dechorionated and anesthetized with 200 mg L^{-1} buffered tricaine (MS-222) prior to the introduction of bacteria. Subsequently, embryos were aligned on an agar plate and injected with 50 CFU of either *C. turicensis, C. muytjensii,* or *E. coli* (range 42 – 66 CFU) in 1-2 nl of a bacterial suspension in DPBS into the yolk sac. The number of CFU injected at 0 hpi was determined by disintegrating and plating 5 embryos individually immediately after microinjection of bacteria (0 hpi) which resulted in an accurate determination of the numbers of CFU actually injected.

Following injections infected embryos were allowed to recover in a petri dish with fresh E3 medium for 15 min. To follow infection kinetics and for survival assays, embryos were transferred into 24-well plates (one embryo per well) in 1 ml E3 medium per well, incubated at 28 °C and observed for signs of disease and survival under a stereomicroscope twice a day. For survival assays after infection, the number of dead larvae was determined visually based on the absence of heartbeat. At each time point, usually 0, 24, 48 and 72 hpi, five embryos were collected and individually treated for bacterial enumeration.

The maximum age reached by the embryos during experimentation was 120 hpf (72 hpi) for which no license is required from the cantonal veterinary office since embryos had not yet reached free feeding stage.

Bacterial enumeration by plate counting

Three larvae were transferred to a separate 1.5-ml Eppendorf tube containing 1 ml DPBS supplemented with 1% Triton X- 100 and disintegrated by repeated pipetting and vortexing for 3 minutes. These were subsequently, serially diluted and 100 µl of the respective mixture was plated onto LB selective plates. Plates were incubated up to 48 h at 37 °C and CFU were determined.

***Mmp-9* silencing in zebrafish embryos by morpholino**

Yolk sacs of one-cell stage zebrafish embryos were microinjected with concentrations of 500, 250, 125, 50 mM translation-blocking zebrafish *mmp-9* morpholino (MMP-9-MO, 5'- CGC CAG GAC TCC AAG TCT CAT TTT G -3') and off-target control human β-globin antisense morpholino (Control-MO, 5'-CCT CTT ACC TCA GTT ACA ATT TAT A-3') (Gene Tools, USA). Some of the morpholino injected embryos were sacrificed at 24, 72 and 96 hpi (= 1, 2 and 3 days post fertilization, dpf).

RNA isolation and quantitative reverse transcription PCR

Forty (infected, non-infected) embryos from each group (*C. turicensis, C. muytjensii*, *E. coli* or mock-infected group) were collected at specified time points and re-suspended in 0.5 ml of the lysis buffer of the RNeasyPlus Mini Kit (Qiagen). The samples were mechanically disrupted for 1 min at 6,500 rpm using lysing bead matrix in MagNA lyser tubes using the MagNA Lyser Instrument (Roche Molecular Diagnostics, Rotkreuz, Switzerland). RNA was isolated from (infected/non-infected) zebrafish embryo and/or bacterial lysates following the RNeasy Plus Mini Kit protocol

(Qiagen). Genomic DNA was removed by using a genomic DNA binding column followed by an on column DNAse I digestion. Ambion's MICROBEnrich kit was used to enrich bacterial RNA from mixed host-bacterial RNA populations which selectively depletes host cell RNA, leaving behind highly enriched bacterial RNA. RNA was eluted in 50 µl of RNAse-free water, and subsequently quantified and quality controlled using the Nanodrop and BioAnalyzer instruments, respectively. The Quantitect Reverse Transcription Kit (Qiagen) was used to reverse transcribe 100 ng of RNA into cDNA. Residual DNA contamination was ruled out in each RNA sample by including a control in which the RT enzyme was omitted. Reverse transcription quantitative-PCR (RT-qPCR) was performed to determine the expression levels of genes in zebrafish and in bacteria injected into zebrafish. Some 2.5 ng cDNA was used to perform quantitative PCR using the primers that are listed in Supplemental Table T2 and SYBR green I kit (Roche Molecular Diagnostics) in the LC480 (Roche Molecular Diagnostics) instrument.

Quantification was performed using the Light Cycler 480 Relative Quantification Software (Roche Molecular Diagnostics). The mRNA levels were normalized using 16S rRNA as reference gene for bacteria and β-actin for zebrafish.

Western blot analysis

Forty embryos from each group (*C. turicensis, C. muytjensii*, *E. coli* or mock-infected group) were collected and resuspended in RIPA lysis buffer with protease inhibitor cocktail (Cell Biolabs Inc, San Diego, USA). The samples were mechanically disrupted for 1 min at 6,500 rpm using lysing bead matrix in MagNA lyser tubes using the MagNA Lyser Instrument (Roche Molecular Diagnostics, Rotkreuz, Switzerland).

The concentration of protein in each sample was determined using Nanodrop Protein A280 protocol. For western blot analysis, 20 µg lysate per sample was suspended in Laemmli Sample Buffer (Biorad, Cressier, Switzerland), and heated to 95 °C for 5 min. Subsequently, samples were subjected to sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) and western blot analysis using MMP-9 rabbit polyclonal primary antibody (Aviva systems biology, San Diego, USA) and HRP-conjugated anti-rabbit IgG secondary antibody using standard techniques (BioRad technical protocol). ImageJ software (National Institute of Health, USA) was used for densitometry determinations to asses fold differences in the protein levels from Western blots.

Statistical analysis

Kaplan Meier survival analysis and statistics for experiments with zebrafish was done with GraphPad Prism 7 (GraphPad Software, United States). Experiments were performed in triplicates.

RESULTS

Expression of *mmp-9* in zebrafish embryos during infection with *Cronobacter turicensis*.

The expression of *mmp-9* was monitored in zebrafish embryos injected with *C. turicensis*, *C. muytjensii* and *E. coli* at 24 and 41 hpi. Experiments with *C. muytjensii* were included as it was reported from previous studies that this strain is an apathogenic member of the *Cronobacter* species[11]. Injection with *C. turicensis*

induces the expression of zebrafish *mmp-9* to a much higher (> 3 fold) extent than the two other bacterial strains at both time points investigated (Figure 1). In addition, expression of the *mmp-9* increases in *C. turicensis* over the course of infection but decreases in infections with the *C. muytjensii* and *E. coli*.

Figure 1: Expression (as determined by qRT-PCR) analysis of bacterial *mmp-9* in zebrafish embryos during infection with the three bacterial strains shown along with experimental controls.

Analysis of MMP-9 protein levels during infection with *Cronobacter and E. coli*

Western blot analysis was performed on zebrafish embryos infected with *C. turicensis* in order to determine the presence of pro-MMP-9 (92-kDa) and its processed form (77-kDa) over the course of infection. As shown in Figure 2, both forms are clearly detected at 6 hpi. However, the intensity of the pro-MMP-9 band appears decrease in intensity whilst the MMP-9 band at-77 kDa increases. This observation suggests the presence of higher amounts of processed/activated MMP-9 at the later time points during infection. The intensity of the β-actin band (control, 42 kDa) remained unaltered over the same time course.

Figure 2: Western blot analysis to detect the presence of pro-MMP-9 and processed MMP-9 at several time points during infection with *C. turicensis*.

In a next step the presence and amount of pro-MMP-9 and the activated form

of MMP-9 in zebrafish embryos injected with *C. turicensis*, *C. muytjensii* and *E. coli* at time point 48 hpi (= 2 dpi) was investigated. Levels of activated MMP-9, as determined by this method and as shown in quantitative data obtained from the Western blot (Figure 3), were significantly lower in embryos injected with *C. muytjensii* and *E. coli*. This result supports the qRT-PCR expression data from Figure 1.

Figure 3: (A): Western blot analysis of pro-MMP-9 and its processed form MMP-9, along with β-actin (control) in embryos injected with *C. turicensis, C. muytjensii and E. coli at* time point 48 hpi. (**B):** quantification of the band for the processed MMP-9 (77 kDa).

Silencing of *mmp-9* in zebrafish embryos.

For a detailed analysis, *mmp-9* morphants were created in zebrafish embryos. In a first step, the amount of morpholino that had to be injected to silence *mmp-9* expression was determined by Western blot analysis using antibodies directed against zebrafish MMP-9 and β-actin (control). Experiments using a nonsense morpholino were carried out to show, that the knock down of *mmp-9* was the result of injection of the MMP-9 morpholino directly into the embryos. In Supplementary Figure S1 results of experiments of embryos injected with varying amounts of morpholino are shown. Both MMP-9 related bands disappear when 500 nm morpholino was injected. This injection dose was used for further experiments.

MMP-9 contributes to Zebra fish model protection against *Cronobacter* infection by controlling bacterial load.

Survival experiments were carried out on wt zebrafish and their isogenic *mmp-9* morphants infected with *C. turicensis*, *C. muytjensii* and *E. coli*. At 1-day post infection (dpi) survival rates of 80 % and 50 % were observed in wt embryos and morphants respectively in infection experiments with *C. turicensis*, suggesting that MMP-9 may function to combat bacterial infection during the early stage of infection. However, zero percent survival was determined in both wt and morphant embryos at 2 dpi (Figure 4). A protective effect of MMP-9 was also observed in experiments using the “apathogenic” *C. muytjensii* strain wherein survival rates dropped from 100 % in wt embryos to 60 % in morphants at 3 dpi (Figure 4). In contrast, survival rates in infections using *E. coli* remained unaltered in both groups of embryos.

Figure 4: Survival data of wt embryos (ZF wt) and *mmp-9* morphants (ZF morphant) shown when infected with *C. turicensis, C. muytjensii* and *E. coli*.

Based on these observations it would be reasonable to hypothesize that MMP-9 may play a role in the killing of the bacterial pathogen at the early stages of infection. This theory was further tested, experimentally by assessing the success or otherwise when using various bacterial doses over the course infection, again with these three bacterial strains. These data are shown in Supplemental Figure S2. During infections with *Cronobacter* strains the bacterial load was increased in *mmp-9* morphants, thus highlighting a role of MMP-9 in killing of *Cronobacter* cells during the

early phase of infections *in vivo*. However, this effect was not observed with *E.* coli indicating that this organism was incapable of growing in zebrafish embryos.

C*. turicensis* Zpx mediates expression of *mmp-9.*

q-RT-PCR experiments with embryos infected with the delta *zpx C. turicensis* mutant clearly showed a reduced expression of *mmp-9*, whereas expression was restored to a certain degree in embryos infected with the *C. turicensis zpx* complemented mutant (Figure 5). Elevated expression of MMP-9 was also observed in embryos injected with *E. coli* carrying the *C. turicensis* wt *zpx* gene *in trans*, thus supporting the latter finding (Figure 5).

Figure 5: Transcriptional expression (as measured by qRT-PCR analysis) of *mmp-9* in zebrafish embryos during infection with *C. turicensis* wt, the delta *zpx* mutant, the mutant complemented with the wt *zpx*, *E. coli* wt and *E. coli* carrying the *C. turicensis zpx* gene as well as controls at 24 hpi.

MMP-9 triggers the expression of the *C. turicensis zpx*

In order to study the possible influence that MMP-9 might exert on *zpx* expression, qRT-PCR assays were performed in WT and *mmp-9* morphants embryos infected with *C. turicensis,* the *C. turicensis* mutant complemented with *zpx* and *E. coli* carrying *C. turicensis zpx* gene *in trans* at 24 hpi. Expression of *zpx* was significantly reduced in morphants during infections with all strains tested (Figure 6). In a control experiment, *zpx* expression was determined when the three strains were

injected into human β-actin antisense (control) morphants. In these experiments *zpx* expression levels were similar to the ones observed in injected wt zebrafish (Supplemental Figure S3).

Figure 6: Transcriptional expression (as measured by qRT-PCR analysis) of *zpx* in *C. turicensis wt*, the *zpx* mutant complemented with the wt *zpx*, as well as *E. coli* carrying the *C. turicensis* wt *zpx* in zebrafish embryos and *mmp-9* morphants at 24 hpi during *in vivo* infection conditions.

MMP-9 negatively affects survival of embryos during infection with *zpx* depleted *C. turicensis*

To further examine the possible interaction of *C. turicensis* Zpx and zebrafish MMP-9, infection experiments were carried out with wt embryos and *mmp-9* morphants which were injected with *C. turicensis* wt, the *zpx* mutant and the complemented mutant as well as *E. coli* carrying the C. *turicensis zpx* gene *in trans*. Survival rates in *mmp-9* morphants were found to be higher (80 %) than in wt embryos (60 %) in infections with the *zpx* depleted *C. turicensis* strain at 3 dpi (Figure 7A). This finding suggested a potential interplay existed between the bacterial Zpx and the host MMP-9. Higher survival rates of zebrafish wt embryos (40 %) *versus* morphants (30 %) were observed in infections with *E. coli* carrying *C. turicensis zpx,* a finding that appears to support our working hypothesis (Figure 7B).

Figure 7: Survival data of wt zebrafish embryos and MMP-9 morphants during

infections with **(A)** *C. turicensis, C. turicensis* delta *zpx*, *C. turicensis* delta *zpx* complemented with the *C. turicensis zpx* and controls. **(B)** *E. coli* Xl1 blue, *E. coli* Xl1 blue carrying the *C. turicensis zpx* gene in trans and controls over 72 hpi.

C. turicensis Zpx processes pro MMP-9 *in vivo*

We hypothesized that the *C. turicensis* Zpx may be involved in the processing of pro-MMP-9 *in vivo*. The results of the respective analysis are depicted in Figure 9. Presence of *C. turicensis* Zpx (in the respective strains) resulted in a stronger band for the processed MMP-9 as analysed by Western blot analysis, which was also confirmed by densidometric measurements of the band representing the processed MMP-9 on the blot using the ImageJ software (National Institute of Health, USA).

Figure 8: (**A)**: Western blot analysis of pro-MMP-9 and processed forms in embryos injected with *C. turicensis*, the delta *zpx* mutant, the delta *zpx* mutant complemented with the *C. turicensis zpx* gene*, E. coli* carrying the *C. turicensis zpx in trans* and controls. (**B):** Densidometric data for the band representing the processed MMP-9 (77-kDa) obtained by analysis using ImageJ software (National Institute of Health, USA).

DISCUSSION

Matrix metalloproteinases (MMPs) have important functions in extracellular matrix (ECM) degradation and tissue repair[15]. MMP-9 has been found to contribute not only to gelatinase activity, but also to leukocyte migration, thus participating in mammalian inflammation and immunity[16,17]. MMP-9 in zebrafish is expressed notably

in the head-kidney and in peritoneal and peripheral blood leucocytes, indicating its role in immune responses[18].

The current study demonstrated the involvement of zebrafish MMP-9 in countering early *Cronobacter* infections in zebrafish embryo models. In the study by Shan et al.[7], migration of macrophages to the site of infection was markedly impaired in *mmp-9* morphants in *Listeria* zebrafish infections resulting in rapid death and higher bacterial loads compared to infections in wt zebrafish. Survival rates of 50 % (morphants) and 80 % (wt) at 1dpi and higher bacterial loads at 24 and 48 hpi during infections with *C. turicensis* wt (Figure 8A, Supplementary Figure S2) suggest a similar mechanism in fighting early stages of infection. Further Shan et al. (2016) suggested activation of MMP-9 *via* a non-proteolytic mechanism as a mechanism to protect against infection. However, it has been shown in other studies that degradation of extracellular matrix proteins such as collagens by MMPs such as MMP-9 may promote migration of macrohages necessary to control infections[19,20]. Thus we hypothesize that MMP-9 is a crucial factor in controlling the bacterial load during the early stages of an infection possibly by recruiting (additional) macrophages in order to kill the intruder thereby preventing bacterial dissemination in the host. However, it has been shown in a study by Eshwar et al.[21] that *C. turicensis* is capable of surviving and replicating in macrophages and it was suggested that macrophages may be used by the pathogens as vehicle to traverse from the site of infection to other parts of the body. The latter feature may explain why *Cronobacter* internalized in macrophages are protected during the later stages of infection while MMP-9 and/or other host components are no longer sufficient to combat infection

372 with *C. turicensis*.

373 In the second part of the study MMP-9 was identified as a substrate of the *C.*
374 *turicensis* zinc metalloprotease Zpx. This protease was originally characterized in *C.*
375 *sakazakii* ATCC 29544^{T} by Kothary et al.[10] and was found to be a member of the
376 metzincin metalloprotease, that possesses collagenolytic activity. Thus, it was
377 suggested that this factor may be responsible for necrosis and extensive cellular
378 destruction in neonates with necrotizing enterocolitis but it may also be involved in
379 allowing the organism to cross the blood-brain barrier. Eshwar et al.[11] reported on the
380 role of this virulence factor in zebrafish embryos infected with *C. turicensis* . In the
381 same study survival data for infections with *C. muytjensii* (*Enterobacter sakazakii*)
382 ATCC5132^{T} was presented showing that it was virtually apathogenic despite the fact
383 that this strain also possesses Zpx which, according to the data presented in the
384 study by Kothary et al.[10] should present a high activity in several *in vitro* assays
385 applied in the study to determine the activity of this protease. However, results from
386 the present study indicated that *C. muytjensii* is hardly, if at all growing/persisting in
387 zebrafish embryos during infection, which may explain the low levels of *mmp-9*
388 expression as well as the low level of processed MMP-9 determined in zebrafish
389 infection experiments.

390 It has already been shown in a previous study that knock out of the *C.*
391 *turicensis zpx* results in enhanced survival rates in wt. zebrafish embryos[11]. Results
392 from the current study showed a similar phenotype also for infections with the *zpx*
393 depleted *C. turicensis* strain in *mmp-9* morphants (Figure 8A). In addition it was
394 observed that *C. turicensis* Zpx induces the expression of MMP-9 and is capable of

processing pro-MMP-9 thus possibly activating its proteolytic properties. Higher survival rates in morphants *versus* wt embryos during infections with the *zpx* depleted *C. turicensis* strain over the whole observation period further supported the hypothesis of an interplay between these two molecules (Figure 8A). We hypothesisze that *zpx* induction of expression and activation of the MMP-9 may lead to uncontrolled action of MMP-9 resulting in additional adverse effects presented by this protease e.g. on extracellular tissues resulting in higher mortality rates. The latter "cumulative, adverse effect" of Zpx and MMP-9 may also account for higher mortality rates in *mmp-9* morphants during infections with wt *C. turicensis* (expressing Zpx) observed at 1dpi. From our results we conclude, that Zpx expressed in *C. turicensis* may interfere with the tightly defined roles exhibited by host MMP-9 to fight infections by these organisms. However, the observation that expression of the bacterial *zpx* is mediated by host MMP-9 was somewhat unexpected and requires further investigation.

Conflict of Interest Statement: The authors declare no conflict of interest.

Supplementary information accompanies the manuscript on the *Emerging Microbes & Infections* website http://www.nature.com/emi

References

1. Tall BD, Chen Y, Yan QQ *et al. Cronobacter*: an emergent pathogen a using meningitis to neonates through their feeds. *Sci Prog* 2014; **97**:154-172.

2. Delclaux C, Delacourt C, D'Ortho MP, Boyer V, Lafuma C, Harf A. Role of

gelatinase B and elastase in human polymorphonuclear neutrophil migration across basement membrane. *Am J Respir Cell Mol Biol* 1996;**14:** 288–295.

3. Opdenakker G, Van den Steen PE, Dubois B *et al.* Gelatinase B functions as regulator and effector in leukocyte biology. *J Leukoc Biol* 2001; **69:** 851-9.

4. Pugin J, Widmer MC, Kossodo S, Liang CM, Preas II HL, Suffredini AF. Human neutrophils secrete gelatinase B in vitro and in vivo in response to endotoxin and proinflammatory mediators. *Am. J. Respir. Cell Mol. Biol* 1999; **20:** 458-464.

5. Dubois B, Starckx S, Pagenstecher A, Oord J, Arnold B, Opdenakker G. Gelatinase B deficiency protects against endotoxin shock. *Eur J Immunol* 2002; **32**: 2163-2171.

6. Renckens R, Roelofs JJ, Florquin S, *et al.* Matrix metalloproteinase-9 deficiency impairs host defense against abdominal sepsis. *J Immunol* 2006; 15; **176:** 3735-41.

7. Shan Y, Zhang Y, Zhuo X, Li X, Peng J, Fang W. Matrix metalloproteinase-9 plays a role in protecting zebrafish from lethal infection with *Listeria monocytogenes* by enhancing macrophage migration. *Fish Shellfish Immunol* 2016; **54:**179-87.

8. Ming D, Moore AG, Bain MA, Breit SN Lyons JG. Activation of macrophage promatrix metalloproteinase-9 by lipopolysaccharide-associated proteinases. *J Immunol* 2002; **168:** 2449-2455

9. Oggioni MR, Memmi G, Maggi T, Chiavolini D, Iannelli F, Pozzi G. Pneumococcal zinc metalloproteinase ZmpC cleaves human matrix metalloproteinase 9 and is a virulence factor in experimental pneumonia. *Mol Microbiol* 2003; 4**9:** 795-805.

10. Kothary MH, McCardell BA, Frazar CD, Deer D, Tall B. Characterization of the zinc-containing metalloprotease (*zpx*) and development of a species-specific detection method for *Enterobacter sakazakii*. *Appl Environ Microbiol* 2007; **73:** 4142–4151.

11. Eshwar AK, Tall BD, Gangiredla J, *et al.* Linking genomo- and pathotype: exploiting the zebrafish embryo model to investigate the divergent virulence potential among *Cronobacter* spp. *PLoS One* 2016; **29:** 11.

12. Fehr A, Eshwar AK, Neuhauss SC, Ruetten M, Lehner A, Vaughan L. Evaluation of the zebrafish as a model to study the pathogenesis of the opportunistic pathogen *Cronobacter turicensis*. *Emerg. Microbes Infect* 2015; **4:** e29.

13. Essers B, Baenzinger O, Huisman TA, Berger C, Moenkhoff M. Neonatal sepsis with *Enterobacter sakazakii* in premature twins, *Swiss Med Wkly* 2006; **136**.

14. Schmid M, Iversen C, Gontia I *et al.* Evidence for a plant-associated natural habitat for *Cronobacter* spp. *Res Microbiol* 2009; **160:** 608-14.

15. Page-McCaw AJ, Werb EZ. Matrix metalloproteinases and the regulation of tissue remodelling *Nat Rev Mol Cell Biol* 2007; **8:** 221-233.

16. Van den Steen PE, Dubois B, Nelissen I, Rudd PM, Dwek RA, Opdenakker G. Biochemistry and molecular biology of gelatinase B or matrix metalloproteinase-9 (MMP-9). *Crit Rev Biochem Mol Biol* 2002; **37:** 375-536.

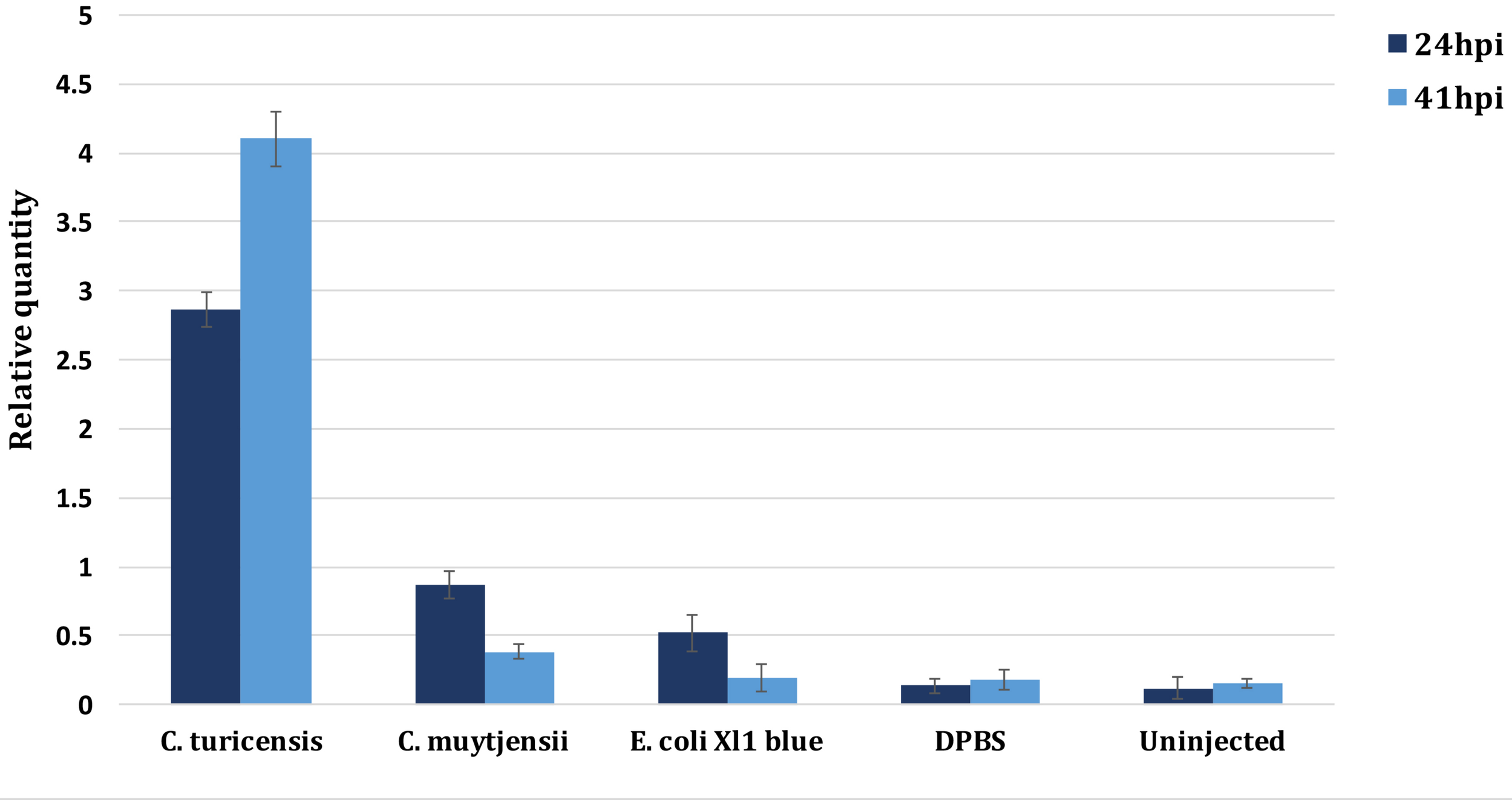
17. Greenlee KJ, Werb Z, Kheradmand F. Matrix metalloproteinases in lung: multiple, multifarious, and multifaceted. *Physiol Rev* 2007; **87:** 69-98.

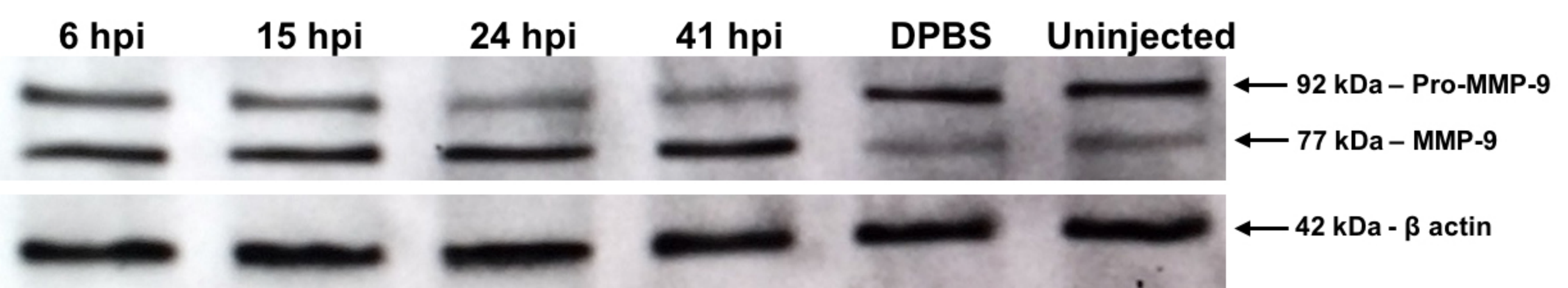
18. Chadzinska M, Baginski P, Kolaczkowska E, Savelkoul HFJ, Verburg-van Kemenade BML. Expression profiles of matrix metalloproteinase 9 in teleost fish provide evidence for its active role in initiation and resolution of inflammation. *Immunology* 2008; **125:** 601-610.

19. Shubayev VI, Angert M, Dolkas J, Campana WM, Palenscar K, Myers RR. TNFa-induced MMP-9 promotes macrophage recruitment into injured peripheral nerve, *Mol Cell Neurosci* 2006; **31:** 407-415.

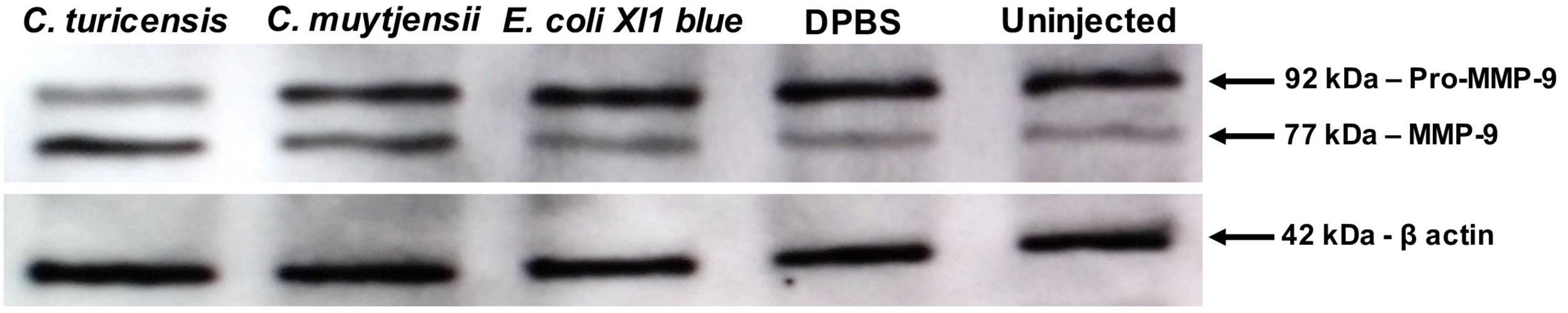
20. Legrand C, Gilles C, Zahm J-M, *et al.* Airway epithelial cell migration dynamics: MMP-9 role in celleextracellular matrix remodeling, *J Cell Biol* 1999; 146:517-529.

21. Eshwar AK, Tasara T, Stephan R, Lehner A. Influence of FkpA variants on survival and replication of *Cronobacter* spp. in human macrophages. *Res Microbiol* 2015; **166:** 186–195.

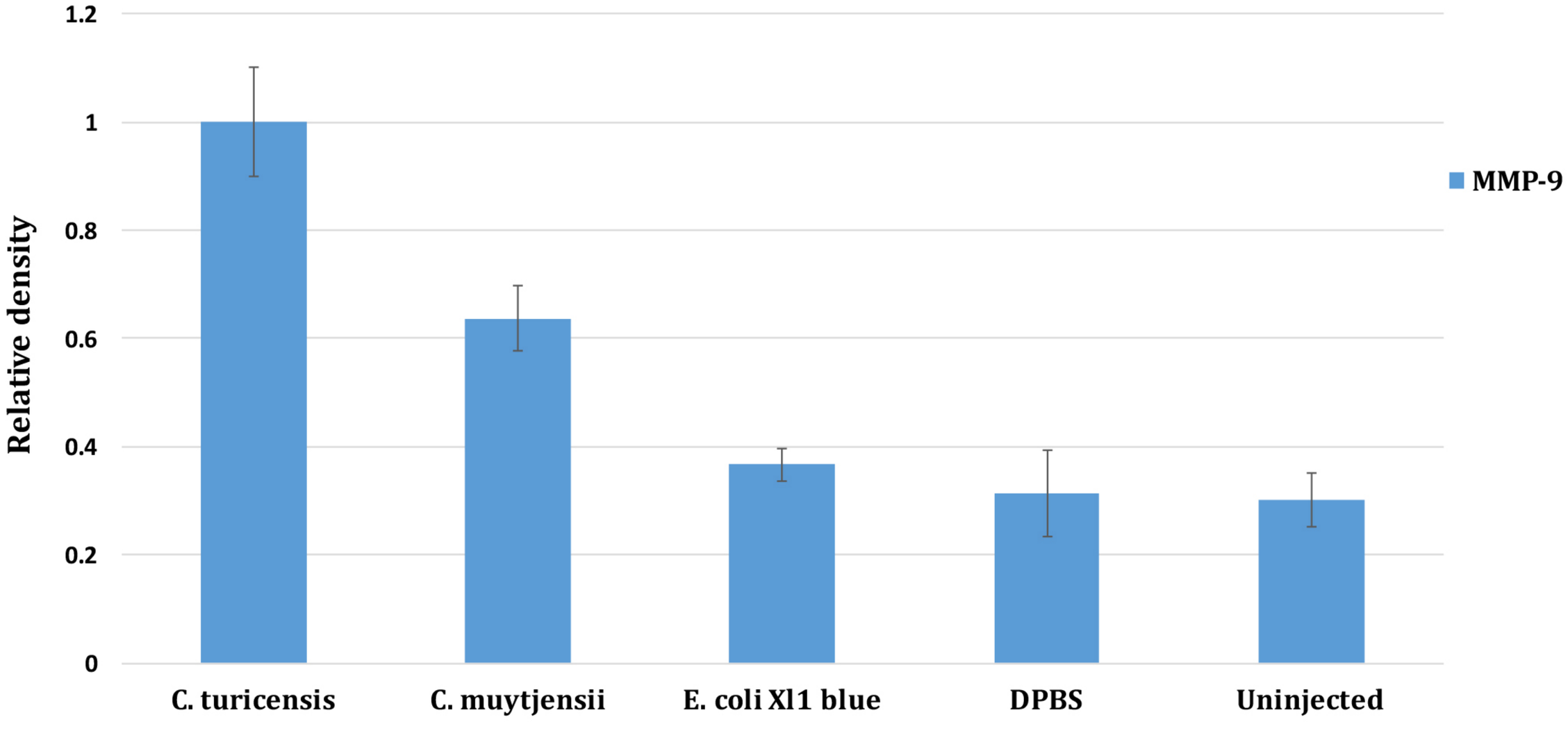

Relative quantity
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
24hpi
41hpi
C. turicensis
C. muytjensii
E. coli Xl1 blue
DPBS
Uninjected

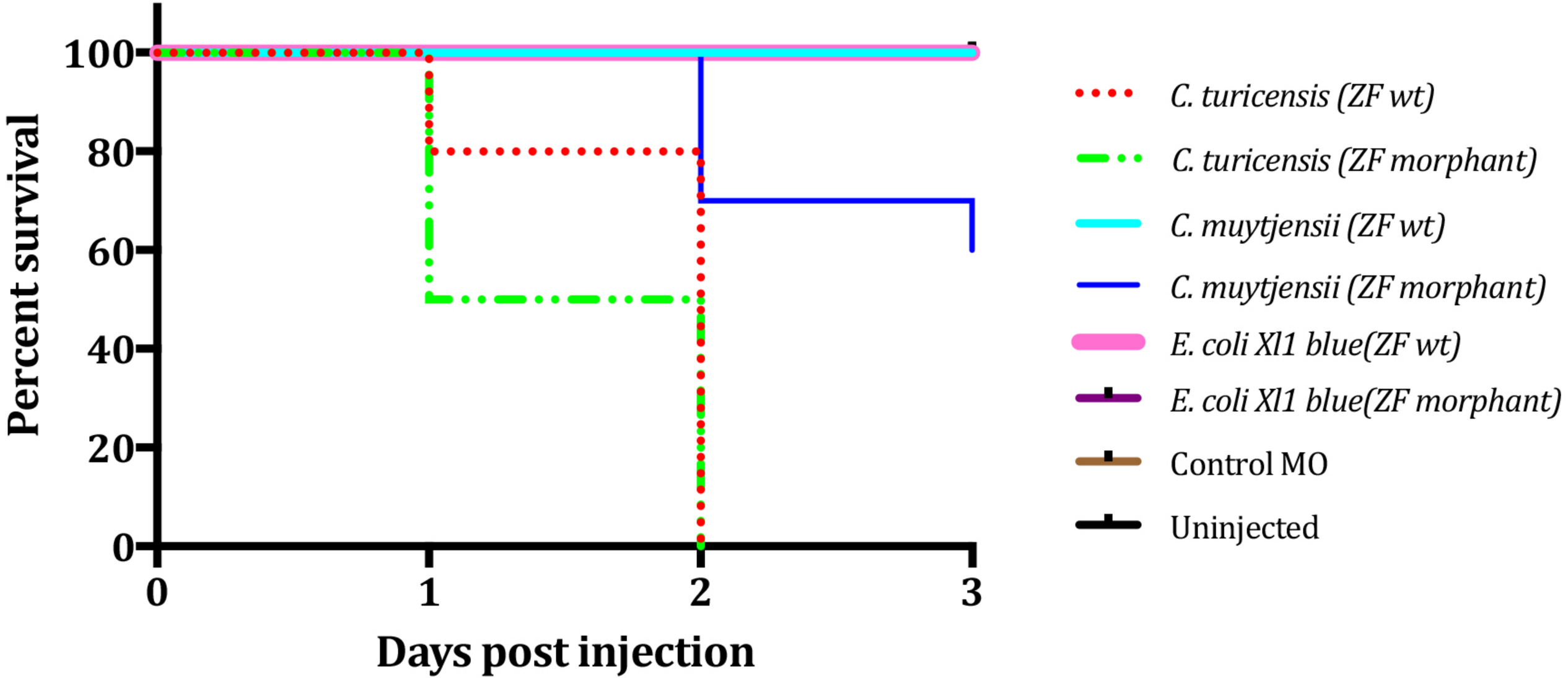

6 hpi
15 hpi
24 hpi
41 hpi
DPBS
Uninjected
92 kDa – Pro-MMP-9
77 kDa – MMP-9
42 kDa - β actin

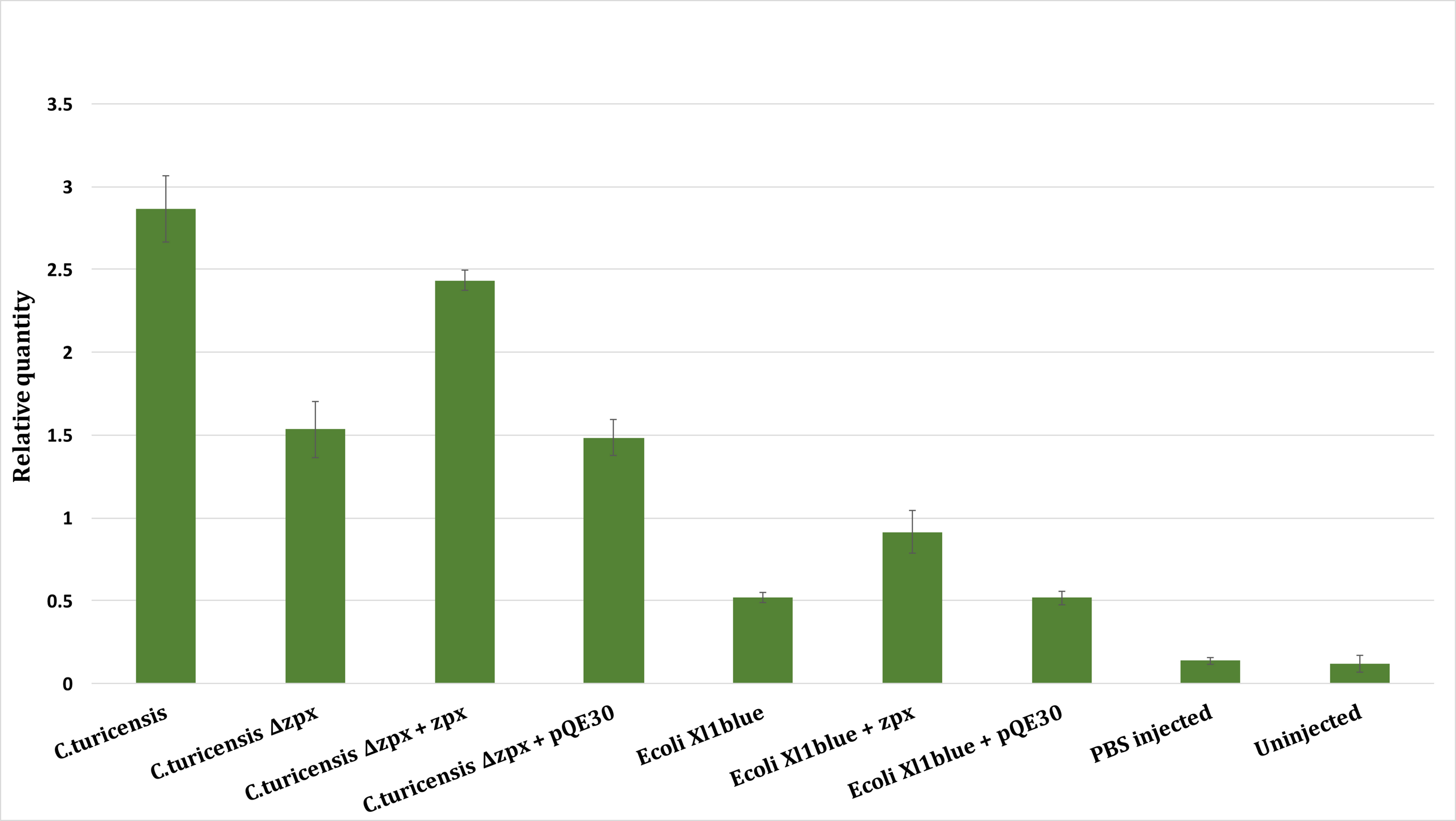
3A

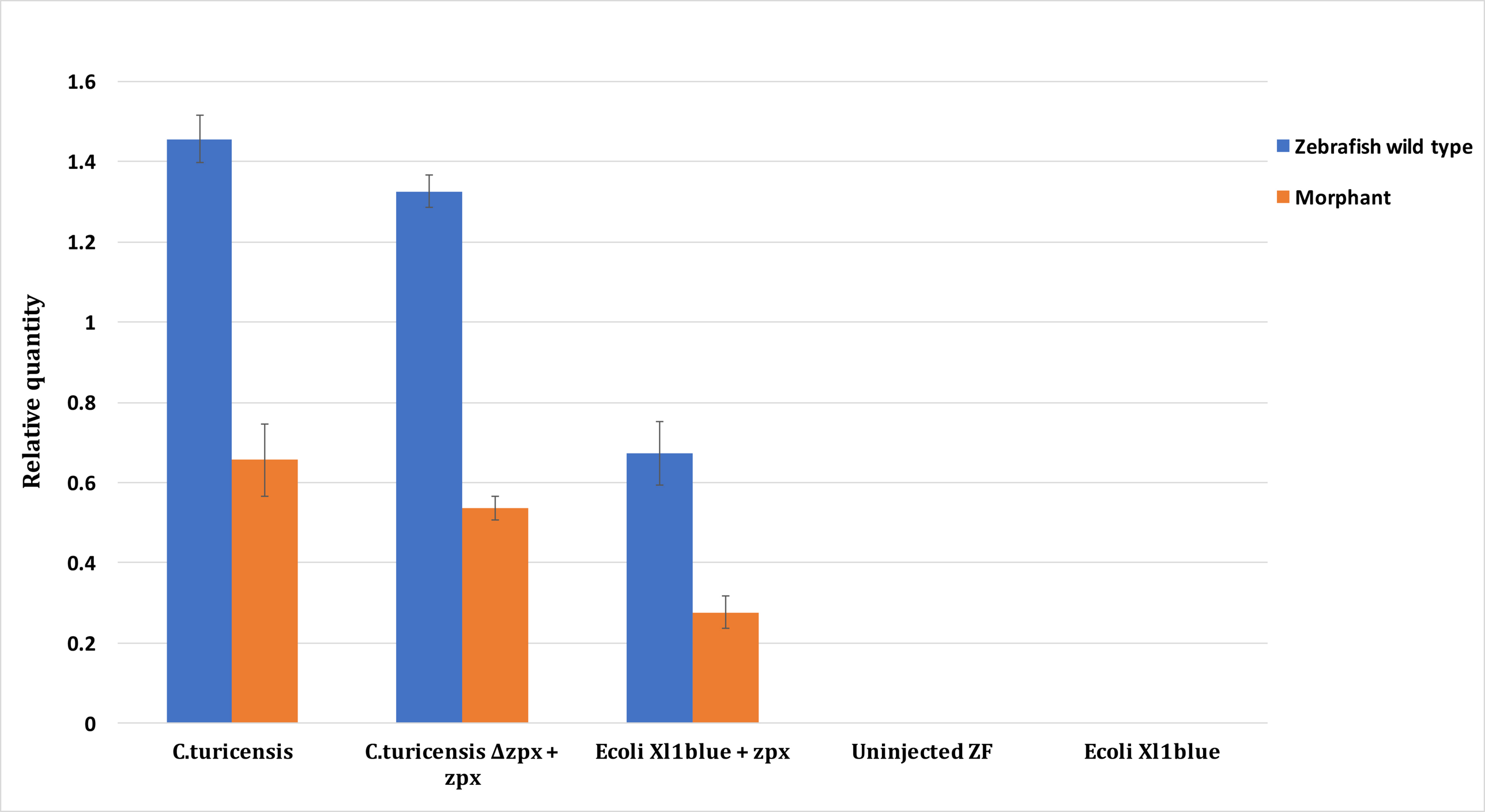

C. turicensis
C. muytjensii
E. coli Xl1 blue
DPBS
Uninjected
92 kDa – Pro-MMP-9
77 kDa – MMP-9
42 kDa - β actin

3B

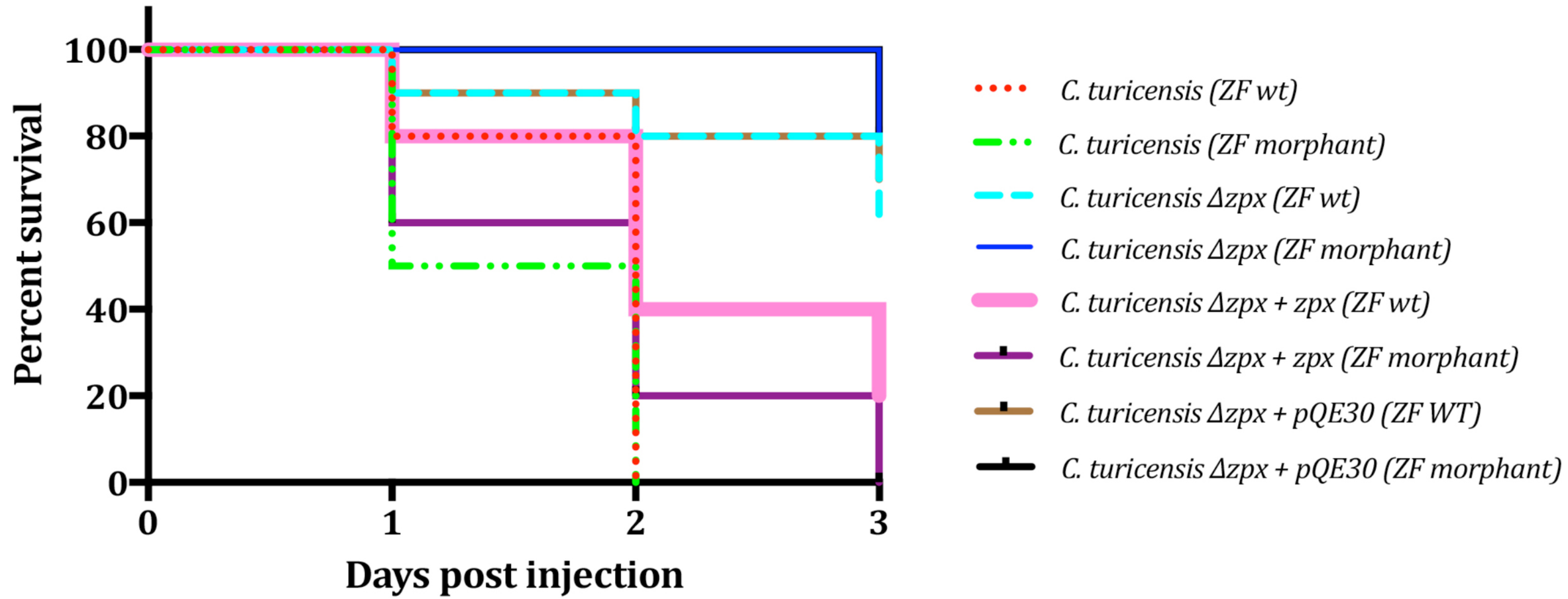

1.2
1
0.8
0.6
0.4
0.2
0
Relative density
MMP-9
C. turicensis
C. muytjensii
E. coli Xl1 blue
DPBS
Uninjected


Percent survival
100
80
60
40
20
0
0
1
2
3
Days post injection
C. turicensis (ZF wt)
C. turicensis (ZF morphant)
C. muytjensii (ZF wt)
C. muytjensii (ZF morphant)
E. coli Xl1 blue(ZF wt)
E. coli Xl1 blue(ZF morphant)
Control MO
Uninjected

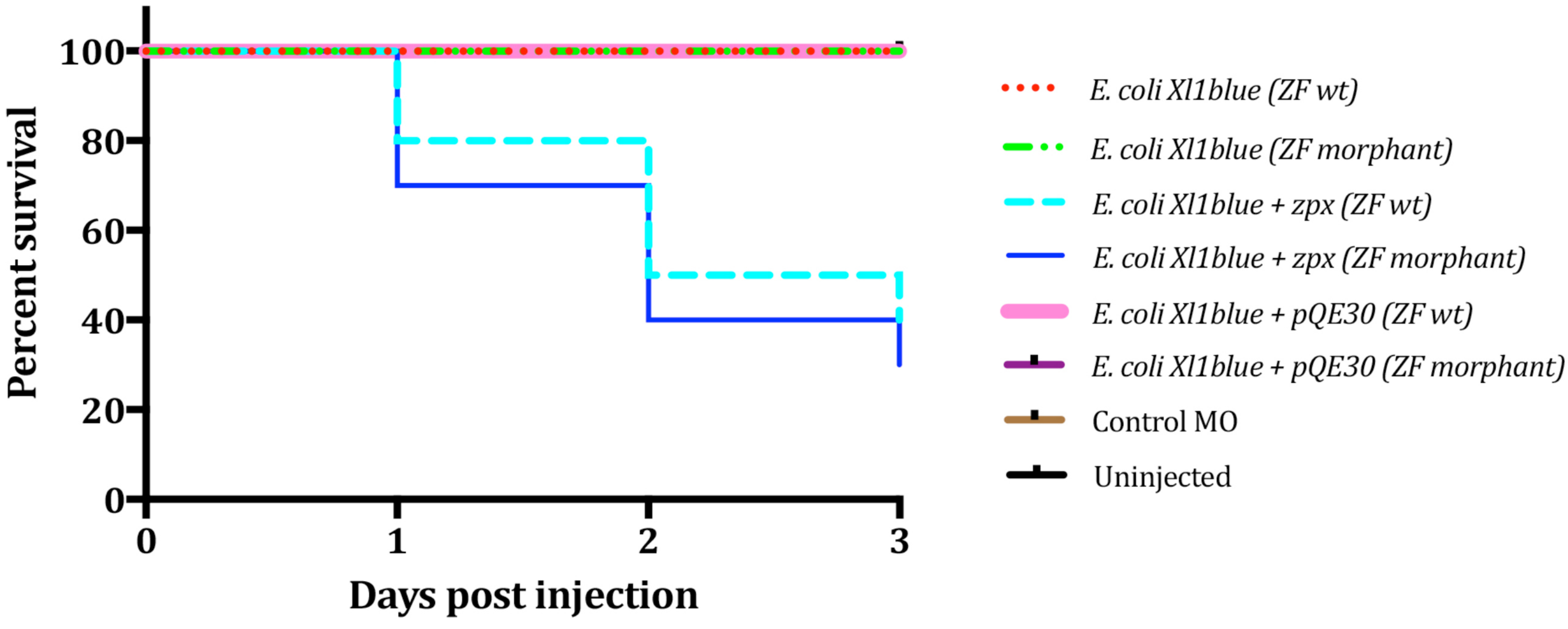

Relative quantity
3.5
3
2.5
2
1.5
1
0.5
0
C.turicensis
C.turicensis Δzpx
C.turicensis Δzpx + zpx
C.turicensis Δzpx + pQE30
Ecoli Xl1blue
Ecoli Xl1blue + zpx
Ecoli Xl1blue + pQE30
PBS injected
Uninjected


1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Relative quantity
Zebrafish wild type
Morphant
C.turicensis
C.turicensis Δzpx + zpx
Ecoli Xl1blue + zpx
Uninjected ZF
Ecoli Xl1blue

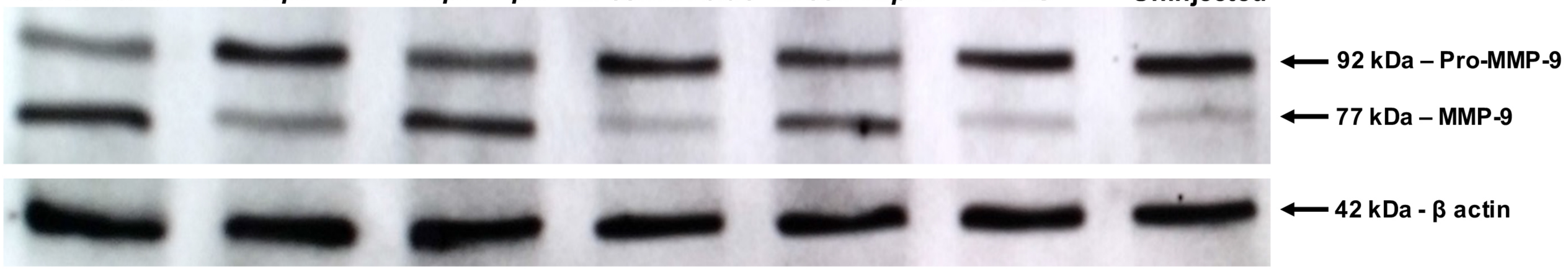
7A


Percent survival
100
80
60
40
20
0
0
1
2
3
Days post injection
C. turicensis (ZF wt)
C. turicensis (ZF morphant)
C. turicensis Δzpx (ZF wt)
C. turicensis Δzpx (ZF morphant)
C. turicensis Δzpx + zpx (ZF wt)
C. turicensis Δzpx + zpx (ZF morphant)
C. turicensis Δzpx + pQE30 (ZF WT)
C. turicensis Δzpx + pQE30 (ZF morphant)

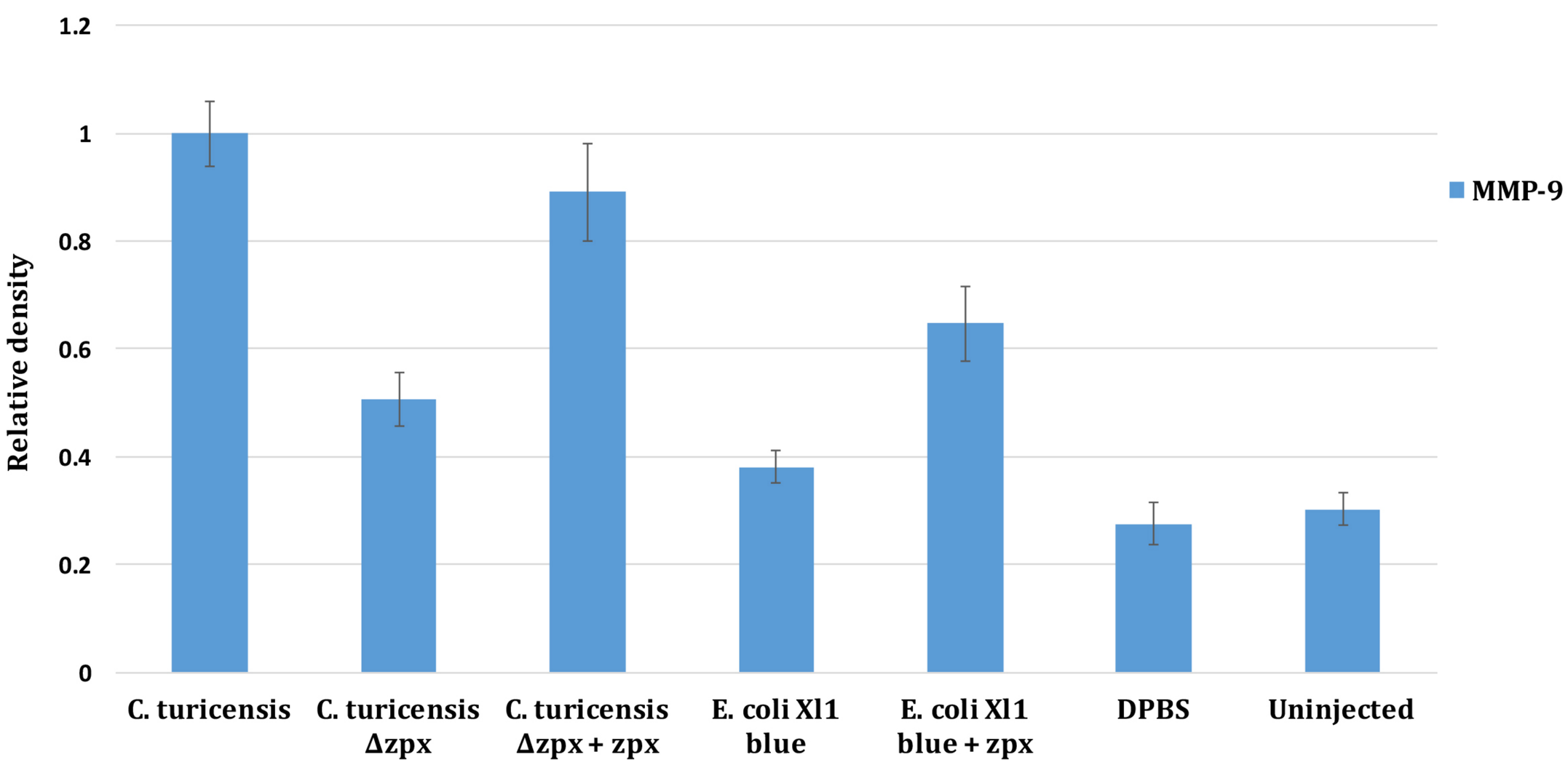
7B


Percent survival
100
80
60
40
20
0
0
1
2
3
Days post injection
E. coli Xl1blue (ZF wt)
E. coli Xl1blue (ZF morphant)
E. coli Xl1blue + zpx (ZF wt)
E. coli Xl1blue + zpx (ZF morphant)
E. coli Xl1blue + pQE30 (ZF wt)
E. coli Xl1blue + pQE30 (ZF morphant)
Control MO
Uninjected

8A


C. turicensis
Δzpx
Δzpx + zpx
E. coli Xl1 blue
E. coli + zpx
DPBS
Uninjected
92 kDa – Pro-MMP-9
77 kDa – MMP-9
42 kDa - β actin

8B


1.2
1
0.8
0.6
0.4
0.2
0
Relative density
MMP-9
C. turicensis
C. turicensis Δzpx
C. turicensis Δzpx + zpx
E. coli Xl1 blue
E. coli Xl1 blue + zpx
DPBS
Uninjected